ORIGINAL ARTICLE

Rats' umbilical-cord mesenchymal stem cells ameliorate mast cells and Hsp70 on ovalbumin-induced allergic rhinitis rats

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ABSTRACT

Aim Allergic rhinitis (AR) is a heterogeneous condition that has been associated with inflammatory responses and is characterized by clinical typical symptoms of nasal itching, sneezing, watery discharge and congestion. Mesenchymal stem cells (MSCs) are multipotent stem cells that have the immunoregulatory ability by secreting various cytokines which potent as a promising therapeutic modality for allergic airway diseases, including AR. The aim of this study was to investigate the effect of rat UC-MSCs on the number of mast cells, the expression of Hsp70 indicated by the nasal symptoms allergic, particularly nasal rubbing in ovalbumin-induced AR rats.

Methods Fifteen male Wistar rats (6 to 8 weeks old) were randomly divided into three groups (control group, sham group, and OVA+MSCs group). OVA nasal challenge was conducted daily from day 15 to 21, and UC-MSCs ($1x10^6$) were administrated intraperitoneally to OVA-sensitized rats on day 21. Nasal rubbing was observed from day 22 to 28. The rats were sacrificed on day 22 and day 28. The nasal cavity tissues were prepared for histological observations.

Results The administration of UC-MSCs could reduce the number of mast cells and the expression of Hsp70 leading to reduction of nasal symptoms allergic, particularly nasal rubbing.

Conclusion Based on this finding, MSCs present a promising immediate curative effect to the inflammatory reaction in AR rats.

Key words: allergic rhinitis, Hsp70, mast cells, MSCs, ovalbumin

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INTRODUCTION

Allergic rhinitis (AR) is a common chronic nasalmucosal inflammatory disease mediated by environmental allergens through IgE with clinicalsymptoms such as nasal rubbing, sneezing, andnasal congestion and can lead to asthma (1,2). IgE antibodies can induce a group of cells including mast cells triggering degranulation of their vesicles and releasing a cascade of inflammatory mediators (histamine) causing localized inflammation, which produces an early phase of AR (2–4). In the innate immune response, histamine promotes the loss of the potential of the mitochondrial membrane and produces reactive oxygen species (ROS), which is marked by an increased heat shock protein 70 (Hsp70) and leads to nasal allergic inflammation in the late phase of AR (5,6).

Nowadays, chemical drugs towards AR are limited to antihistamines, anti-leukotrienes and intranasal corticosteroids, which only alleviate allergic symptoms but fail to regulate the allergic reaction (2,7,8). Infrequently, these drugs have side effects, such as headache, throat irritation, and nasal dryness (8). Thus, the development of more safe and effective therapeutic agents is needed. On the other hand, mesenchymal stem cells (MSCs), the multipotent cells, exhibit a strong immunomodulation potential via their interaction with T lymphocytes, B lymphocytes, natural killer (NK) cells, and dendritic cells (DC) and have the ability to modulate immune systems, which potent as a promising therapeutic modality for allergic airway diseases (1,3,7). Taken together, mast cells and Hsp70 could be a major mediator of AR and may be used as disease biomarkers. The MSCs have been studied extensively as a potential anti-inflammatory treatment in many diseases associated with inflammation and the immune system (1,7,9,10); however, their use in treating AR specifically relates to mast cells and Hsp70 still needs further investigations.

The MSCs are multipotent stem cells in the form of spindle-like shape which can be derived from bone marrow, adipose, umbilical cord, dermis, synovial membrane, and gingiva (11,12). The immunoregulatory ability of MSCs have resulted in its wide usage as a therapy in allergic disease (7,9,13). Previous studies revealed that MSCs can modulate dendritic cell maturation, suppress natural killer cell function, and inhibit the allogeneic T cell response by altering the cytokine secretion profile of dendritic cells and T cells induced by an allogeneic immune reaction (9,14–17). The MSCs in allergy in vitro and in vivo studies of asthma using bone marrow demonstrated that MSCs enhanced Th1, iNOS, and IL-10 immune responses (18). The MSCs can inhibit IgE, eosinophil, IL-4 and IL-13 and chemotaxis factors in vitro studies (19), and inhibited B cells via IFN-ɣ inhibition. Reportedly, adipose-derived stem cells can be localized in the nasal mucosa and decrease the eosinophils (7), suppress Th2 by inducing Treg (20), reduce Th2-mediated cytokine (21), and inhibit IL-4 and IL-5 (22). Stem cells from the tonsils reduce symptoms of allergic rhinitis and inhibit IgE production (4). Also, recent studies demonstrated that bone marrow mesenchymal stem cells inhibited allergic nasal symptoms, including rubbing and sneezing and reduced the number of eosinophils in the nasal mucosa via decreased Th2 cytokine (IL-4, IL-5, and IL-13) levels and regulatory cytokines (IL-10) (23).

Histamine released by mast cells not only modulates dendritic cell response but also induces iNOS expression, nitrite oxide (NO) production, promotes loss of mitochondrial membrane potential, and produces ROS (1,2,24). In RA, oxidative stress manifests when the production of free radicals, such as ROS accumulate in the nasal mucosa and lead to an increase of Hsp70 expressions (5,25). Several recent studies have also suggested that mast cells and Hsp70 play crucial roles in the pathogenesis and process of allergic reactions during AR (2, 28–31). In addition, a recent study also revealed that Hsp70 shows an extensive assortment of immunological influences in addition to its cytokine effects, and it can be considered a signal that alerts our defence system of an impending emergency (26). However, studies about the use of MSCs to treat AR, especially those related to mast cells, Hsp70, and nasal rubbing are still limited.

The aim of this study was to investigate the effect of MSCs treatment on AR via mast cells and Hsp70, the effect of rats UC-MSCs on the number of mast cells, the expression of Hsp70 indicated by the nasal symptoms allergic, particularly nasal rubbing in ovalbumin-induced AR rats.

MATERIALS AND METHODS

Materials and study design

In this post-test only control group study design was conducted in Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang from January – June 2021. Fifteen male Wistar rats (6 to 8 weeks old) were raised in a controlled environment, with a regular 12-hour light-dark cycle and unrestricted access to OVA-free food and water. All mice that were used in this study were handled according to an approved protocol and were randomly divided into three groups (control group, sham group, and OVA+MSCs group). For the induction of allergic asthma, the mice were first sensitized with an intraperitoneal (i.p.) injection of 1 mg of OVA (Sigma-Aldrich, St. Louis, MO, USA) and 2.25 mg aluminum hydroxide gel (alum adjuvant; Thermo Fisher Scientific, Waltham, MA, USA) in 100 µL of sterile saline on days 0, 1 and 10. After systemic sensitization, the mice were locally challenged by intranasal (i. n.) instillation of 50µg/10µL of OVA into their nostrils from days 15 to 21. UC-MSCs ($1x10^6$) were administrated intraperitoneally to OVA-sensitized rats on day 21, while the control group received saline (NaCl) injection. Nasal rubbing was observed from day 22 to 28. The rats were sacrificed on day 22 and day 28 and the nasal cavity tissues were prepared for histological observations.

The study was approved by the Ethic Committee of Universitas Sumatera Utara. (142/KEP/USU/2020).

Methods

Isolation of MSCs. Rat UC-MSCs were isolated from a 19-day pregnant female rat. Briefly, donor rats were anesthetized, and the abdomens were dissected out. Under sterile conditions, the umbilical cord (UC) was collected and washed in phosphate-buffered saline (PBS). The UC artery and vein were removed, then the UC was cut into lengths of 2–5 mm using a sterile scalpel. The sections were then distributed evenly in T25 flasks using Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% PBS, 100 IU/ml penicillin/streptomycin (GIBCO, Invitrogen), then incubated at 37 °C with 5% CO_2. The medium was renewed every 3 days, and the cells were passaged after reaching 80% confluence. UC-MSCs at passages 4–6 were employed for the following experiments.

Characterization of MSCs. The MSCs were grown in a 24 well plate (1.5×10^4 cells/well) with a standard medium containing DMEM (Sigma-Aldrich, Louis St, MO), enriched with 10% FBS (Gibco™ Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco Invitrogen, NY, USA) at 37 °C, 5% CO_2 and ≥95% humidity. After 80% confluent, the adipogenic protocol was initiated. For adipogenic differentiation, the growth medium was switched to Human Mesen Cult Adipogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore). The medium was changed every other day and at day 35 the cultures were stained with Oil Red O and observed under the microscope.

The MSCs were analysed for specific surface marker expression by flow cytometry. Briefly, the cultured cells were incubated in the dark with primary antibodies mouse anti-human CD29, mouse anti-human CD90, and mouse anti-human Lin negative (CD45/CD31) followed by the secondary conjugated antibody. MSCs were stained with a specific antibody for 30 minutes at 4°C, examined with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA), and analysed with BD Accuri C6 Plus software (BD Biosciences, San Jose, CA, USA) (27).

Nasal rubbing observation. Observation of clinical symptoms was carried out by 2 observers on the same subject for 10 minutes. Each mouse was put into a transparent cage, then labelled on each cage to determine the mouse code. Furthermore, the rats were observed for nasal rubbing behaviour for 10 minutes of observation time.

Animal tissue collection. After the RA mice were treated with MSCs, the mice were sedated using a mixture of ketamine anaesthetic (80-100 mg/kg) and xylazine (5-10 mg/kg) administered intramuscularly. The skull tissue of each group was taken by neck decapitation after the rat was confirmed to be dead, then the skin part of the skull was removed, and the skull was fixed using 10% neutral buffered formalin as much as 70% of the organ volume (Bio-Optica Milano S.p.A), which was then carried out by the paraffin method tissue processing.

Toluidine blue staining. The nasal cavity tissue was deparaffinized by immersing the samples in xylene (Merck-Millipore, Australia) 1 and 2 for 5 minutes, respectively. Then the rehydration process was carried out by immersing the slides in absolute 1, absolute 2 ethanol, 90% ethanol, 80% ethanol, 70% ethanol, 70% ethanol (Merck-Millipore, Australia) for 3 minutes each, then washed in running water for 5 minutes, then soaked in distilled water for 5 minutes. Dip into the toluidine blue working solution (Sigma, St. Louis, MO, USA). Next, dehydration was done by dipping the sample slide on the metal staining rack into 70% ethanol, 80% ethanol, 96% ethanol, absolute 1, absolute 2 ethanol, for 15 seconds each, then the clearing process by dipping the slides in xylene 1, 2 and 3 each for 3 minutes, then mounting the tissue with entellant and observed in a light microscope.

Immunohistochemistry. The nasal cavity paraffin-embedded sections (5 µm) were deparaffinized, extensively washed in PBS, blocked for 1 h with donkey serum, and incubated overnight at 4°C with a rabbit anti-mouse Hsp70 polyclonal antibody (1:100 dilution; Thermo Fisher, Rockford, IL) in a humidified chamber. The slides were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:200) from Trekkie Universal Link (Starr Trek Universal-HRP Detection Kit) for 60 min at room temperature as described previously (28). Immunostaining was developed with 1:400 diaminobenzidine (DAB). Mayer hematoxylin (Bio-Optica Milano S.p.A) was used as the counterstain. Representative images were obtained using an Olympus bright-field microscope, the low-magnification images were taken with a 4x objective, and the high-magnification images were taken with a 400x.

Statistical analysis

A statistical analysis with a significance level (α) of 0.05 or 5% used the data normality test using the Shapiro Wilk test. Immunofluorescent images were taken and analysed using Image J software (National Institutes of Health, USA). The data in this study were normally distributed and homogeneous, then the differences between groups were analysed using one-way ANOVA and continued with a least significant difference (LSD) comparison post hoc test.

RESULTS

The MSCs culture after the fourth passage showed cells adhering to the bottom of the flask with spindle-like cell morphology under microscopic observation (Figure 1A). The adipogenic differentiation test was performed by inducing MSCs using a special adipogenic medium that formed adipocytes (Figure 1B). Flow cytometry analysis showed that MSCs were able to express CD90 (99.8%), CD29 (94.2%), and lack of CD45 (1.6%) and CD31 (6.6%) (Figure 1C).

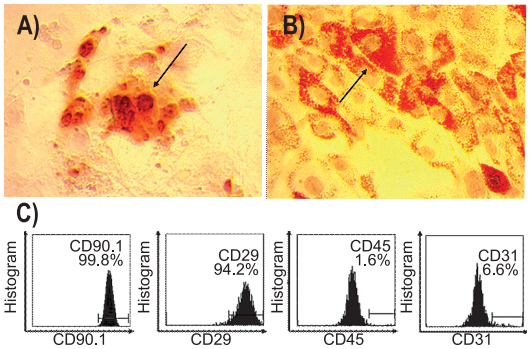

Figure 1. Characteristics of MSCs. A) The spindle-like morphology of MSC is pointed to by the arrow on the microscope observation (200x magnification); B) The differentiation test shows that there are fat deposits on the MSCs marked in red using oil red O staining (magnification 400x); C) Flow cytometry analysis of the expression of CD90, CD29, CD45, and CD31

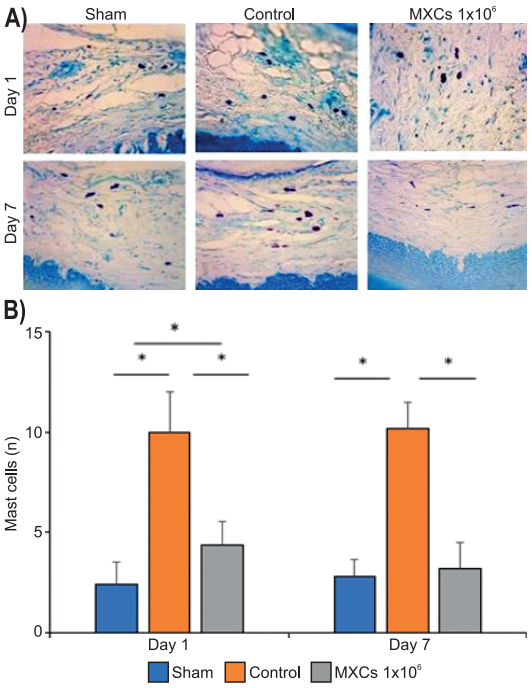

Figure 2. The number of mast cells in rats for three groups after MSCs treatment on the first and seventh day. A) Histological observation using toluidine blue staining (400X magnification); B) The number of mast cells in each treatment; *p<0.05

Based on the observation of the number of mast cell fractions (Figure 2) on the first day, the number of mast cells in the treatment group showed a significant decrease ($p<0.05$) compared to the control group, 4.4±1.14 and 10±2, respectively. On the seventh day of observation, the mast cell count of the treatment group reduced to 3.2±1.3 compared to the control group, 10.2 ± 1.3.

HSP70 expression on the first day of observation in the treatment group had a lower number than that of the control rats' group, 50.56±4.64% and 68.24±5.93%, respectively, and on the seventh day 41.52 ± 5.83% and 68.08 ± 5.56%, respectively (Figure 3).

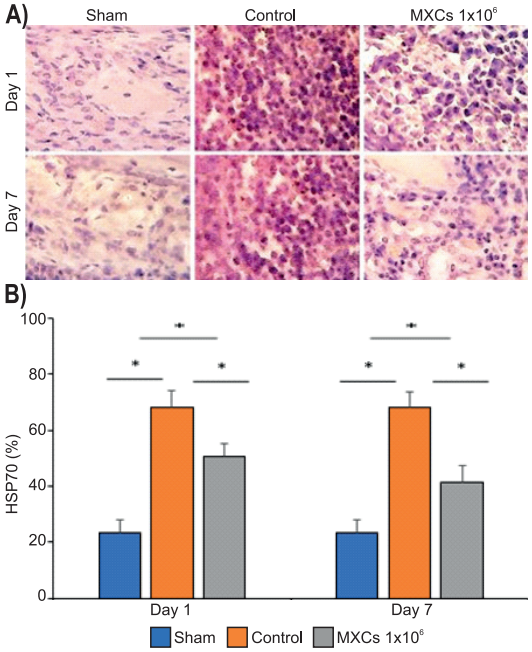

Figure 3. Expression of HSP70 in the nasal cavity tissue in three groups used the immunohistochemical staining method with HSP70 antibodies. A) HSP70 immunohistochemical staining results in each treatment (400x magnification); B) Quantification of HSP70 expression in each treatment group on day 1 and day 7; *p<0.05

Administering MSCs on the first and seventh day was able to significantly reduce the number of nose rubs, 3.8±1.92 and 2.4±1.56 n/10 minutes, compared to the control group, 6.9±2.30 and 6.8± 2.84 n/10 minutes, respectively (Figure 4).

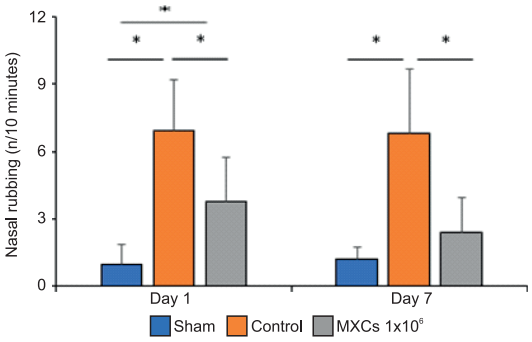

Figure 4. The effect of MSCs on the nasal rubbing symptom in OVA-induced allergic rhinitis mice. The time of nasal rubbing of day 3 and day 7 were measured for 10 min after the last intranasal challenge. The time of nasal rubbing for each day; *p<0.05

DISCUSSION

This study aimed to determine the effect of UC-MSCs in AR rats, investigating the effect of the rat UC-MSCs on the number of mast cells, the expression of Hsp70 indicated by the nasal symptoms allergic, particularly nasal rubbing in ovalbumin-induced AR rats. We have shown that the administration of UC-MSCs could ameliorate AR rats by reducing the number of mast cells and regulating the expression of Hsp70 indicated by reducing nasal symptoms allergic, particularly nasal rubbing.

Recent studies have suggested that T helper type 2 (Th2) cells and mast cells play crucial roles in the pathogenesis and process of allergic reactions during AR (1,6,29)the levels of Hsp70, Hsp90, interleukin (IL. Th2 cells mediate the activation and maintenance of the allergens and secrete a population of Th2 cytokines, such as interleukin 4 (IL-4) (30)there is a high frequency of allergen-specific interleukin-4 (IL-4. Then, more T helper cells are recruited into the allergic response and promote the synthesis and secretion of immunoglobulin E (IgE) from B lymphocytes (2,30) sneezing, watery discharge and congestion. The intention of this review is to illustrate key concepts of the pathogenesis of rhinitis. Imbalance in innate and adaptive immunity together with environmental factors is likely to play major roles. In allergic rhinitis, initial allergen exposure and sensitization involves antigen-presenting cells, T and B lymphocytes and results in the generation of allergen-specific T cells and allergen-specific IgE antibodies. On re-exposure to relevant allergens, cross-linking of IgE on

mast cells results in the release of mediators of hypersensitivity such as histamine and immediate nasal symptoms. Within hours, there is an infiltration by inflammatory cells, particularly Th2 T lymphocytes, eosinophils and basophils into nasal mucosal tissue that results in the late-phase allergic response. Evidence for nasal priming and whether or not remodelling may be a feature of allergic rhinitis will be reviewed. The occurrence of so-called local allergic rhinitis in the absence of systemic IgE will be discussed. Non-allergic (non-IgE-mediated. Once IgE binds to the high-affinity receptor (FcεRI) on the surface of mast cells, special IgE responses to antigens are switched on (2). Subsequently, the binding of IgE - FcεRI activates mast cells, which leads to a degranulation response and the secretion of allergic mediators, including histamine, and results in the infiltration of inflammatory cells, followed by acute or chronic inflammation of the nasal mucosa (2,3,23,29,31).

The accumulating data by several recent studies have demonstrated that the action of MSCs on the immune system and responses via secreting various molecular factors, particularly tumour growth factor-b1 (TGF-β1), interleukin 10 (IL-10), and prostaglandin E2 (PGE2) (1,7,9,32)AR, AR + Montelukast, and AR + MSCs. Mast cells, one of the major effector cells in allergic reactions, play a central role in innate immunity in the pathogenesis of AR by secreting prestress mediators such as histamine, tryptase, and IL-4 via degranulation (20,21,33). Based on several previous studies, PGE2 secreted in large amounts by MSCs is correlated with higher efficacy to EAE inhibition, collagen-induced arthritis mitigation, and mixed lymphocyte reaction suppression leading to inhibit the degradation of mat cells in AR (31,32,34). A similar finding in an allergic disease murine model also revealed that administration of MSCs suppressed both the infiltration and degranulation of mast cells, which was mediated by PGE2 and TGF-β1 secreted by MSCs (7) while the allergic symptoms could be alleviated. Mesenchymal stem cells (MSCs. In addition, a recent study also demonstrated that TGF-β secreted by hUCB-MSCs inhibits TNF-α secretion by mast cells by suppressing ERK/STAT3 signalling and mast cell degranulation (35). In this study, since the number of mast cells after administration of UC-MSCs decreased, we propose that MSC-driven immunomodulation contributes to inhibiting mast cell degradation in reducing hypersensitive in AR via PGE2 and TGF-β1.

Previous reports suggested that anti-Hsp70 levels were correlated with higher levels of total IgE and IL-4 in asthmatic patients shown by increased levels of Hsp70 in nasal lavage fluid in AR patients compared with non-AR patients (26)protein degradation, and transport. Elevated levels of Hsp70 have been found in the sputum, serum, and bronchoalveolar lavage (BAL. In line with these findings, the results of our study also show the regulated expression of Hsp70 after administration of MSCs was confirmed histologically by light microscopy, which showed that the nasal mucosa was near normal with decreased Hsp70 expression. In AR, histamine, a hypersensitivity mediator, released by degranulated mast cells not only regulates dendritic cell response but also influences iNOS expression, nitrite oxide (NO) production, increases impairment of mitochondrial membrane potential, and generates oxidative stress such as ROS accumulate in the nasal mucosa and lead to an increase of Hsp70 levels (1,5,8,21,22). The result of this study is also supported by a previous study which reveals that Hsp70 shows an extensive assortment of immunological influences in addition to its cytokine effects, and it can be considered a signal that alerts our defence system of an impending emergency (26)protein samples from four STZ-DM and four AMC rat corpora tissues were prepared independently and analyzed together across multiple quantitative two-dimensional gels using a pooled internal standard sample to quantify expression changes with statistical confidence. A total of 170 spots were differential expressed among the four experimental groups. A subsequent mass spectrometry analysis of the 170 spots identified a total of 57 unique proteins. Network analysis of these proteins using MetaCore suggested altered activity of transcriptional factors that are of too low abundance to be detected by the two-dimensional gel method. The proteins that were down-regulated with diabetes include isoforms of collagen that are precursors to fibril-forming collagen type 1; Hsp47, which assists and mediates the proper folding of procollagen; and several proteins whose abundance is

controlled by sex hormones (e.g. CRP1 and A2U. Interestingly, Hsp70 levels are affected by stress response times. A previous study showed that 48 h post-stress, Hsp70 expression levels rose to reach their maximum level and decreased at 120 h post-stress (36), which supports this hypothesis. Thus, we predict that MSCs can regulate the stress response due to hypersensitivity to AR through the regulation of Hsp70 expression.

A limitation of this study is that we did not examine histamine secreted by mast cells as a sign of degranulation. In addition, we also did not analyse the ROS levels as a stress response indicator in the AR rats. Therefore, understanding the role of PGE-2 and TGF-β1 secreted by MSCs to regulate histamine expressions associated with ROS levels remains to be explored.

In conclusion, rat's UC-MSCs ameliorate mast cells, Hsp70, and nasal rubbing on ovalbumin-induced AR rats. Based on this finding, MSCs present a promising immediate curative effect to the inflammatory reaction in AR rats.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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